

AD _____

Award Number: DAMD17-02-1-0043

TITLE: PSA Converts Parathyroid Hormone-Related Protein (PTHrP)
from an Osteolytic to an Osteoblastic Factor: Role in
Bone Metastasis

PRINCIPAL INVESTIGATOR: John M. Chirgwin, Ph.D.

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, VA 22904

REPORT DATE: December 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

20040527 143

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE December 2003		3. REPORT TYPE AND DATES COVERED Annual (1 Dec 2002 - 30 Nov 2003)
4. TITLE AND SUBTITLE PSA Converts Parathyroid Hormone-Related Protein (PTHrP) from an Osteolytic to an Osteoblastic Factor: Role in Bone Metastasis			5. FUNDING NUMBERS DAMD17-02-1-0043	
6. AUTHOR(S) John M. Chirgwin, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Virginia Charlottesville, VA 22904 E-Mail: jc3qb@virginia.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Original contains color plates: ALL DTIC reproductions will be in black and white				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) Prostate cancer metastases cause disorganized new bone formation, despite expressing the bone-destructive factor, PTHrP. We report a molecular basis for this paradox: residues 6-9 [LMDK] of ET-1 and PTHrP residues 8-11 [LHDK] share sequence similarity. PTHrP is cleaved at residues 22 and 23 by the serine protease, prostate-specific antigen (PSA). The fragments generated by PSA are too short to activate the PTH1 receptor but stimulate new bone by activating the ETA receptor. We tested PTHrP peptides on bone formation in 4-day neonatal mouse calvariae. PTHrP(1-16) (100nM) caused a 2.5-fold stimulation of new bone area (p<0.001). Osteoblast numbers were correspondingly increased. The response was equivalent to ET-1 (100nM). PTHrP(1-20) (25nM) also increased bone formation. The actions of PTHrP(1-16 & 1-23) were blocked by ABT627 (0.01mM), a selective ETAR antagonist. We found a strong bone anabolic response to PTHrP(1-23) (1nM; p<0.01 vs control), while PTHrP(1-34) instead caused extensive osteolysis. Structural mimicry of ET-1 by PTHrP peptides provides a molecular basis for the osteoblastic phenotype of PTHrP-positive prostate cancer bone metastases. ETAR antagonists should be effective against PTHrP fragments, in treating osteoblastic bone metastases due to prostate cancer.				
14. SUBJECT TERMS Bone metastasis, ET-1, PSA, PTHrP, osteoblastic metastasis				15. NUMBER OF PAGES 20
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

PSA converts parathyroid hormone related protein (PTHrP) from an osteolytic to an osteoblastic factor: role in bone metastasis

DAMD17-02-1-0034

John M. Chirgwin, Ph.D., University of Virginia

TABLE OF CONTENTS:	page
INTRODUCTION	3
BODY	4
KEY RESEARCH ACCOMPLISHMENTS	8
REPORTABLE OUTCOMES	8
CONCLUSIONS	9
REFERENCES	10
APPENDICES	13-19

INTRODUCTION:

Bone metastases are a major source of morbidity and mortality in patients with advanced cancers of the breast and prostate (Mundy, 2002). Parathyroid hormone-related protein, PTHrP, is established as the major known causal agent in osteolytic bone metastases (Guise et al, 1996; Guise, 1997). These metastases are characterized by net destruction of bone in the vicinity of tumor cells and are commonly caused by breast cancers (Guise & Chirgwin, 2003a). By contrast, prostate cancers, which share with breast cancers a propensity to metastasize to skeletal sites, usually cause osteoblastic metastases, lesions that are characterized by net formation of disorganized new bone of poor mechanical quality. In these metastases it has been observed clinically that bone resorption is increased even more than in breast cancer osteolytic metastases. It is also known that prostate cancers usually express the same osteolytic factor, PTHrP (Guise & Chirgwin, 2003b). However, an understanding has been lacking of why prostate cancer metastases are usually osteoblastic rather than osteolytic, despite their expression of PTHrP. Our DoD Idea proposal offered a novel molecular basis to explain this paradox. PTHrP fragments are released by the action of prostate-specific antigen, PSA, which is a chymotrypsin-like serine protease. When these observations were published by Cramer et al and Iwamura et al in 1996, it was thought that the 23-amino acid fragment was inactive, since 1-34 is the minimum fragment length required to activate the PTH type 1 receptor efficiently. In 2001, however, Schluter et al observed that a short fragment of PTHrP stimulated cardiomyocyte contraction by binding to the endothelin type A receptor [ET_AR] on the cells, possibly via a short peptide homology between ET-1 residues 6-9 [LMDK] and PTHrP residues 8-11 [LHDK]. In light of this unexpected observation, we hypothesized that in prostate cancers, PSA could cleave PTHrP into novel ligands of the ET_AR (Figure 1). We have recently established an animal model of osteoblastic metastasis that identifies ET_AR ligands as causal agents of skeletal pathology (Yin et al, 2003).

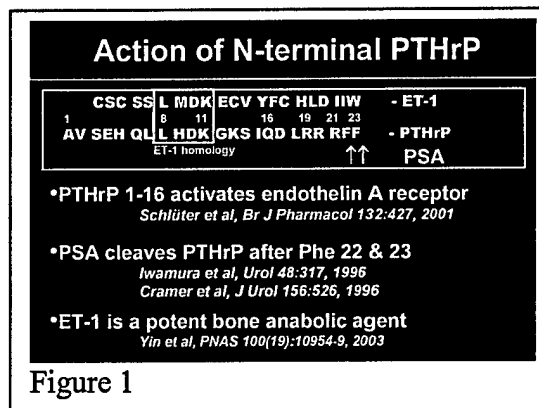


Figure 1

We have now established that PTHrP 1-23 is a potent bone anabolic agent, with maximal bone-stimulatory activity at 1 nM. The effects of this fragment are blocked by the selective ET_AR agonist, ABT-627, Atresentan. This compound is effective in an animal model (Yin et al, 2003) and has completed Phase II clinical trials, in which it was effective at decreasing skeletal-related events in men with advanced, hormone-refractory prostate cancer (Carducci et al, 2003; Nelson et al, 2003). In addition, PTHrP- neutralizing antibodies are now in clinical trials in cancer patients (Sato et al, 2003). This humanized antibody recognizes the N-terminus of the molecule and was originally shown, prior to its humanization, to be effective in decreasing cancer bone metastasis by our Co-investigator (Guise et al, 19996).

In October 2003, I moved, along with my colleagues Drs. K.S. Mohammad and T.A. Guise, from the University of Texas Health Science Center in San Antonio, to the University of Virginia in Charlottesville. This has been a highly rewarding move. The UVa Cancer Center has been extremely supportive with an extensive start-up package. UVa is a major center of prostate

cancer research and has many world-class researchers who focus on basic signaling mechanisms in prostate cancer cells. The move was, however, not facilitated by the previous institution. The office of Grants Management at UTHSCSA was extremely recalcitrant to transfer the grant from UT to UVA, and the award notice was not received by my new institution until October 2003, but I have been able to back—date the activation of this award to 04/01/2003. Thus, the actual funded period covered by this progress report is 04/01/03 – 12/01/03, rather than a full 12 month period. There is thus also a 6-month period in which this research was (involuntarily) not supported by the DoD award. As part of the process of transferring the award from UT to UVA, a revised statement of work (SOW) was submitted in 02/2003. The proposed revised performance dates now differ by no more than one month from reality.

BODY:

Work is reported according to the **revised (2/2003) SOW**, which is included in the Appendix.

For Specific Aim 1

Task 1: Reclone prohK2 & proPSA cDNAs into pcDNA vectors, test DNAs by transient transfections into 293 cells and assay as in task 4, below (months 1-3). **Previously completed.**

Task 2: Isolate stable single cell lines with DNAs from task 1 (months 4-12). **Previously completed for proPSA.** Cell lines for hK2 remain to be isolated (months 1-9).

Task 3: Isolate stable double (hK2+PSA) cell line with DNAs from task 1 and hK2 stable clone from task 2 (months 13-24; now months 6-21).

As referenced in the revised SOW, recent data suggest that cleavage of PTHrP in the PC3 cell line may result in growth inhibition. This seems to be the case and we have so far been unsuccessful in isolating stable clones that express active hK2 (which would cleave PTHrP at arginine residues 19-21 (Figure 1). Thus, an additional section to task 3 has been added. The Isaacs' laboratory has published (Denmeade et al, 2003) detailed characterization of a series of prostate cancer cell lines for their expression of both hK2 and PSA. These lines are LAPC-4, CWR22Rv1, MDA PCA-2b, LNCaP, and C4-2B (derived from LNCaP as a bone-metastatic variant, Thalmann et al, 1994; Wu et al, 1994)), all of which are available to us through the resources of the NCI-supported UVA Cancer Center. We propose to assay these cell lines for PTHrP expression and secretion by three assays. First RNAs will be prepared from the 5 cell lines or xenografts and analyzed by standard RT-PCR for PTHrP gene expression. Second, conditioned media from the cells will be assayed for content of intact PTHrP with the Nichols Institute two-site IRMA. Third, the same media samples will be assayed with a single-site competition RIA, which employs a high-affinity antibody that recognizes the amino-terminus of PTHrP (residues 1-7), from Phoenix, Inc, which has been used successfully by our UVA colleague, Dr. S.J. Parsons. This assay will detect all cleaved forms of PTHrP. We expect that at least some of these cell lines, which make high amounts of active PSA and hK2, may cleave PTHrP efficiently. Media from these lines will be negative with the Nichols ELISA and positive with the Phoenix RIA.

Task 3a: Screen 5 cell lines for PTHrP expression (months 1-9). If none are positive, we will transfect a PTHrP expression DNA (already available and tested) into one of the hK2+, PSA+ lines. Resultant clones would then be screened with the three assays for PTHrP.

TABLE 1

Prostate Cancer Cell Line	PSA	N-term PTHrP	Intact PTHrP	Ratio N-terminal to Intact PTHrP
PC3, wt	0	10.7	9.1	1.2
LNCaP, wt	193	32.3	0.57	57
LNCaP, dox-reg	185	61.7	1.54	40
LAPC4	trace	138.6	1.88	73
C4-2B	165	34.8	0.91	38
PC3, AR+ v1	0	0	0.37	0
PC3, AR+ v2	61	0	8.96	0

PSA expressed as pg/ml/10⁵ cells/48hrs. N-terminal PTHrP expressed as pM/10⁵ cells/48hrs (Phoenix single-site competition RIA). Intact PTHrP expressed as pM/10⁵ cells/48hrs (Nichols two-site IRMA). Media collected serum-free from subconfluent cultures. Dox-reg are LNCaPs stably expressing dox-inducible regulatory system. Data from cells treated with doxycycline. PC3 AR+ are PC3s stably transfected with wt androgen receptor. V1 and v2 are independent clones. Dox-reg, LAPC4, and PC3 AR+ cells grown in presence of synthetic androgen R1881.

Task 3b: Isolate PTHrP+ clones for a prostate cancer hK2+, PSA+ cell line (months 10-21).

Tasks 3a & b are now largely complete. The results are shown in **Table 1**. It is likely that some of these cell lines also express ET-1, which would substantially confound any results found in vivo; so we are presently assaying these same samples for ET-1 peptide content before proceeding to animal experiments. The current data suggest that LNCaP, or its bone-metastatic, androgen independent derivative C4-2B (Thalmann et al, 1994; Wu et al, 1994) are the best candidates for the cell line to be used in the animal experiments, since they express PSA and show a high ratio of N-terminal to intact PTHrP, suggesting substantial cleavage of PTHrP.

Task 4: Screen clones from tasks 2&3 for enzymatic activity and protein expression (months 6-12, 18-24, now months 1-21). *These experiments are underway.*

Task 5: Immuno-affinity purify PTHrP fragments from media of cell lines from tasks 2&3 and analyze fragments by HPLC/mass spectrometry (months 13-18, 25-30, now months 9-15, 25-30). Analysis of fragments from cell lines newly identified from additional subtasks 3a & 3b now included. *We expect to commence this task in 01/2004.*

For Specific Aim 2):

Task 6: Prepare synthetic peptides (months 1-3). **Previously completed.**

Task 7 Test synthetic peptides on osteoclast formation in marrow cultures (months 4-9). **Previously completed.**

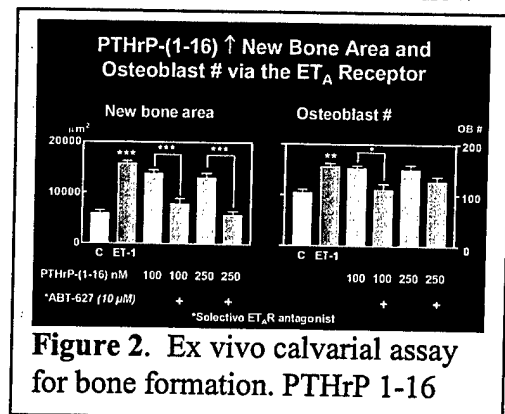
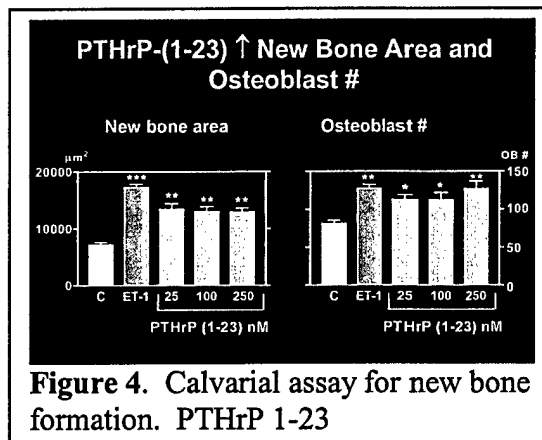
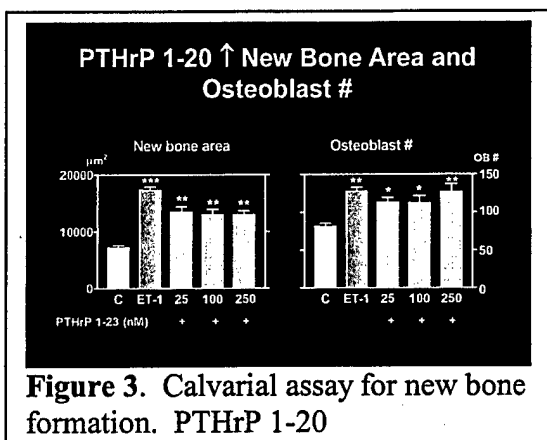
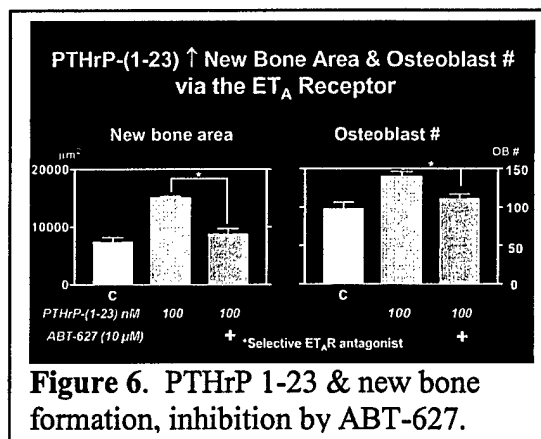
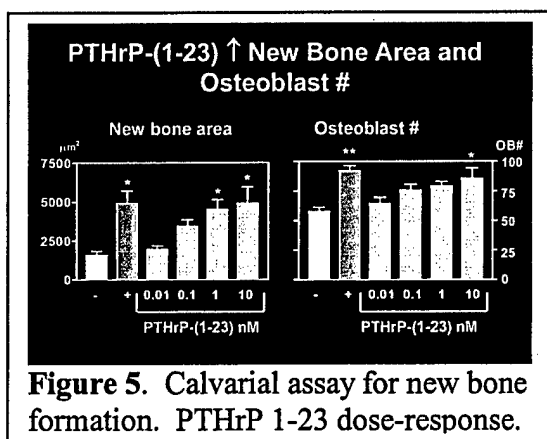


Figure 2. Ex vivo calvarial assay for bone formation. PTHrP 1-16



Task 8: Test synthetic peptides on new bone formation in calvarial cultures (months 6-24). **Now completed.** The results for 1-16, 1-20 and 1-23 and the effects of ABT-627 to block the responses are shown in **Figures 2-6**. **Figure 7** shows representative histology of calvarial bones treated with PTHrP 1-23 and PTHrP 1-23 + ABT-627.



Additional section to task 8. Neonatal mouse calvarial organ cultures are slow and expensive. Now that we have demonstrated the essential correctness of the original hypothesis in organ culture, we will obtain more accurate dose response data using a well-characterized mouse pro-osteoblastic cell line, MC3T3-E1. Twenty-three published papers have tested the ET-1 receptor responsiveness of these cells, and others have shown PTHrP responsiveness (McCauley et al, 2001). Thus, the cells express receptors for both peptides and respond to them. The cells will differentiate into mature osteoblastic cells in culture when treated with ascorbate and form mineralized nodules, as well as express late markers, such as osteocalcin. We will carry out detailed dose responses of the peptides using these cells. We are presently determining the most convenient marker of differentiated response to assay (such as VEGF promoter-luciferase activity (Wang et al, 2002), alkaline phosphatase secretion, or osteocalcin production.). *Experiments underway.* We are presently testing a variety of different osteoblastic sublines. We have also completed Affymetrix gene array analysis for the endothelin targets in mouse osteoblastic cells, which identify approximately 30 additional targets. These are now being validated.

PTHrP-(1-23) Stimulated New Bone Formation: Inhibition by ABT-627

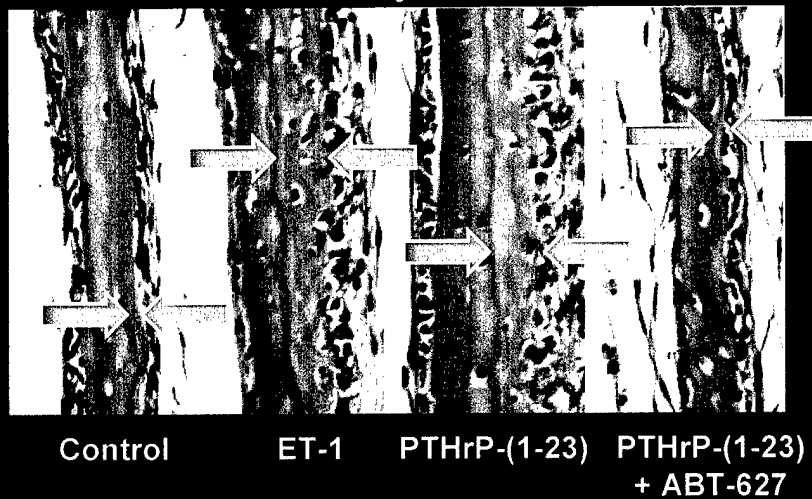


Figure 7. Calvarial assay for new bone formation. Histology from treatment with PTHrP 1-23

Task 8a: Test peptides on MC3T3-E1 cells (months 10-24). *These experiments will be initiated in the next funding period.*

Task 9: Perform tasks 7, 8, and 8a on samples from task 5 (months 19-20, 31-32, now months 9-15 & 37-30).

For Specific Aim 3):

Task 10: Carry out pilot animal experiment for PC3/ev and PC3/hK2 cell lines from Aim 1) in metastasis model with 8 mice (months 15-20, now months 10-15). *We have carried out a pilot animal experiment* with the androgen receptor (AR) –re-expressing PC3 cell lines, shown in **Table 1**. The bone phenotype in these animals will be determined in 2004. The AR-transfection increased survival and decreased the area of bone lesions, however, since these cells do not express significant PSA or have increased expression of N-terminal PTHrP fragments, we do not believe that these cell lines are worth pursuing in the context of the work proposed here.

Task 11: Carry out full animal experiment for the final four cell lines from Aim 1 in metastasis model (n=12 mice/group) and intramuscular tumor growth rate model (n=6 mice/group) with 72 mice total (months 25-30, now months 25-30). Alternatively, this task could be carried out with one of the cell lines identified in task 3a or 3b, which we now believe to be much more likely. As discussed above, we expect to use an intratibial injection model, either with LNCaP cells or the bone metastatic variant C4-2B. Parental LNCaP cells cause mixed osteoblastic/osteolytic lesions over the course of 5-6 weeks with a take rate of just over 50% (Corey et al, 2002), although these authors report that the cells in bone stained positively for ET-1 expression. We expect that C4-2B cells would form bone lesions more rapidly and efficiently, but their production of ET-1, relative to parental LNCaP cells, has not been reported to our knowledge. We will determine this missing datum in 01/2004. LNCaP cells in vitro express extremely low amounts of ET-1 (Grant et al, 1997; Granchi et al, 2001). *We expect to initiate these experiments in the spring of 2004.* The selected prostate cell line (PSA-positive and producing high concentrations of PTHrP fragments shorter than 1-34 and low concentrations of endothelin-1 and longer PTHrP molecules) will be tested in the intra-tibial model, with which we have experience. Animals will be treated with ABT-627 as described (Yin et al, 2003) or PTHrP-neutralizing

mouse monoclonal antibody as described (Kakonen et al, 2002). We have drug and purified mAB available.

Task 12: Carry out detailed histomorphometric analysis of the bones and soft tissues of the 8 mice from task 10 (bones only, months 21-24, now months 16-21) and the 48 metastasis model mice (48+288 bone histology blocks) from the previous task (months 31-34, now months 30-33).

Task 13: Perform statistical analysis of data from animal experiments and prepare manuscripts for publication (months 11-12, 23-24; 35-36, now months 3-6, 20-21 and 32-33).

KEY RESEARCH ACCOMPLISHMENTS:

- PTHrP fragments shown to stimulate new bone formation.
- Effects of PTHrP fragments shown to be via the endothelin A receptor
- Effects of endothelin-1 on osteoblastic metastases published in an animal model (Yin et al, 2003).
- Dose-response of new bone formation to PTHrP 1-23 shown to extend to at least 1 nM.
- A series of prostate cancer cell lines identified that express both PTHrP and active PSA. Preliminary data obtained on conversion of PTHrP into N-terminal fragments by these cell lines.

REPORTABLE OUTCOMES:

We submitted our initial results to three international meetings during 2003. In all three cases the work was selected for oral presentation. **Abstracts for these three presentations**, with dates, titles, and locations of the meetings, are included in the appendices.

We have initiated **two new collaborations** to accelerate the work proposed. The laboratory of Masashi Yanagisawa (the discoverer of endothelin), Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX, is currently testing our PTHrP peptides for binding to and activation of the cloned endothelin A and B receptors. These are technically very difficult experiments, since ET-1 binding to its receptor is essentially irreversible and the assays require highly specialized expertise (Kedzierski & Yanagisawa, 2001). Simultaneously, we are collaborating with the laboratory of Thomas Gardella at Massachusetts General Hospital to determine peptide-binding parameters to the PTH type 1 receptor. Again this is a highly specialized technique, in which Dr Gardella is a world expert (Gensure et al, 2003.) As soon as the binding data to the cloned receptors are obtained, a manuscript summarizing our findings will be prepared and submitted.

CONCLUSIONS:

We have been fully successful to date. Our experiments *ex vivo* establish that PTHrP 1-23 is a potent bone-anabolic agent, while our parallel experiments establish that tumor-produced ligands for the ET A receptor are causal agents in osteoblastic bone metastases (Yin et al, 2003), **Figure 8**.

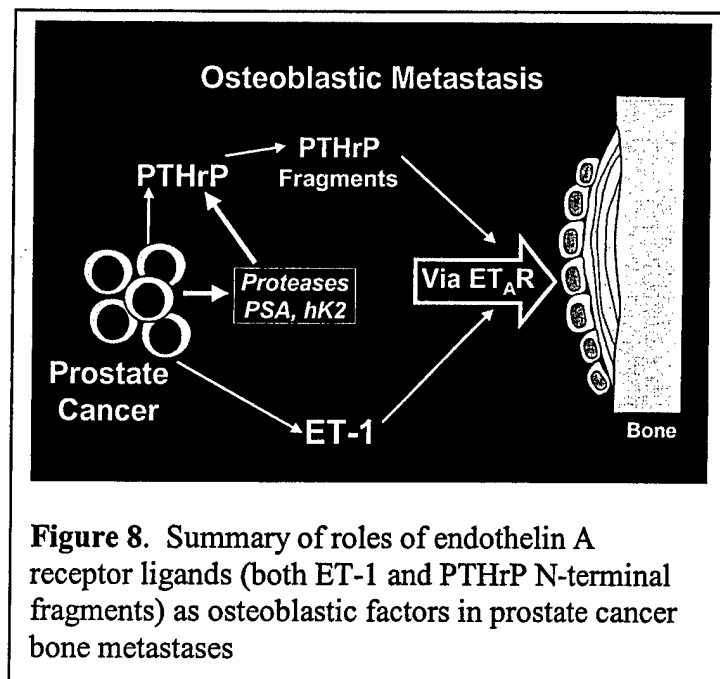


Figure 8. Summary of roles of endothelin A receptor ligands (both ET-1 and PTHrP N-terminal fragments) as osteoblastic factors in prostate cancer bone metastases

We continue to work on developing a human prostate cancer cell line that makes PTHrP N-terminal fragments by the proteolytic action of PSA on intact PTHrP (139 aa and larger). This has turned out to be a challenging project, but we have identified a number of PSA+ cell lines that secrete large amounts of N-terminal PTHrP but low amounts of longer forms. If any of these cell lines are low producers of ET-1, they will be useful candidates to test in an animal model. We have also established the intra-tibial injection model for testing osteoblastic responses to cell lines (such as LNCaP derivatives) that do not form metastases when inoculated into immunocompromised mice by the intracardiac route.

We expect to identify a useful PTHrP-fragment secreting cell line in the 2004 grant period and to commence animal experiments. This is a crucial experiment, since it will test the physiological significance of what are presently *in vitro* observations. Detailed ligand binding experiments are not proposed in this grant but these are now underway with expert collaborators in the fields of ET receptors and PTHrP receptors. These results should substantially strengthen the molecular basis of our observations.

Particularly exciting to us is the current progress in clinical treatments that target the N-terminus of PTHrP and the ET A receptor in cancer (Mundy, 2003). These are described in the INTRODUCTION. They hold out the possibility that these therapeutic interventions could be rapidly applied to the animal model, which we are currently developing. In addition, our results provide insights into a physiological role for PSA (and hK2) in prostate cancer bone metastases. They suggest that inhibitors of PSA would not benefit patients with PTHrP+ PSA+ bone metastases, but would rather change the bone response from osteoblastic to osteolytic. We have recently discovered published data that neprilysin, another protease secreted by prostate cancers, also cleaves PTHrP at residue 23 (Ruchon et al, 2000) and may play an endogenous role in normal bone. Neprilysin inactivates endothelin-1 and appears to be decreased with prostate cancer progression (Nelson & Carducci, 2000). It may be that changes in protease expression (PSA, hK2, neprilysin, et al) alter the processing of a set of bone active factors such as PTHrP and ET-1 and contribute to the phenotype of bone metastasis characteristic of prostate cancer.

REFERENCES:

- Carducci MA, Padley RJ, Breul J, Vogelzang NJ, Zonnenberg BA, Daliani DD, Schulman CC, Nabulsi AA, Humerickhouse RA, Weinberg MA, Schmitt JL, Nelson JB (2003). Effect of endothelin-A receptor blockade with atrasentan on tumor progression in men with hormone-refractory prostate cancer: a randomized, phase II, placebo-controlled trial. *J Clin Oncol.* 21:679-89.
- Corey E, Quinn JE, Bladou F, Brown LG, Roudier MP, Brown JM, Buhler KR, Vessella RL (2002). Establishment and characterization of osseous prostate cancer models: intra-tibial injection of human prostate cancer cells. *Prostate* 52:20-33.
- Cramer SD, Chen Z, Peehl DM (1996). Prostate specific antigen cleaves parathyroid hormone-related protein in the PTH-like domain: inactivation of PTHrP-stimulated cAMP accumulation in mouse osteoblasts. *J Urol.* 156:526-31.
- Denmeade SR, Sokoll LJ, Dalrymple S, Rosen DM, Gady AM, Bruzek D, Ricklis RM, Isaacs JT (2003). Dissociation between androgen responsiveness for malignant growth vs. expression of prostate specific differentiation markers PSA, hK2, and PSMA in human prostate cancer models. *Prostate* 54:249-257.
- Gensure RC, Shimizu N, Tsang J, Gardella TJ (2003). Identification of a Contact Site for Residue 19 of Parathyroid Hormone (PTH) and PTH-Related Protein Analogs in Transmembrane Domain Two of the Type 1 PTH Receptor. *Mol Endocrinol.* 17:2647-2658.
- Granchi S, Brocchi S, Bonaccorsi L, Baldi E, Vinci MC, Forti G, Serio M, Maggi M (2001). Endothelin-1 production by prostate cancer cell lines is up-regulated by factors involved in cancer progression and down-regulated by androgens. *Prostate* 49:267-277.
- Grant ES, Brown T, Roach A, Williams BC, Habib FK (1997). In vitro expression of endothelin-1 (ET-1) and the ETA and ETB ET receptors by the prostatic epithelium and stroma. *J Clin Endocrinol Metab* 82:508-513.
- Guisse TA (1997). Parathyroid hormone-related protein and bone metastases. *Cancer.* 80(8 Suppl):1572-1580.
- Guisse TA, Chirgwin JM (2003a). Transforming growth factor-beta in osteolytic breast cancer bone metastases. *Clin Orthop.* 415 Suppl:S32-38.
- Guisse TA, Chirgwin JM (2003Bb). Role of bisphosphonates in prostate cancer bone metastases. *Semin Oncol.* 30:717-723.
- Guisse TA, Yin JJ, Taylor SD, Kumagai Y, Dallas M, Boyce BF, Yoneda T, Mundy GR (1996). Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. *J Clin Invest.* 98:1544-1549.

- Iwamura M, Hellman J, Cockett AT, Lilja H, Gershagen S (1996). Alteration of the hormonal bioactivity of parathyroid hormone-related protein (PTHrP) as a result of limited proteolysis by prostate-specific antigen. *Urology*. 48:317-325.
- Kakonen SM, Selander KS, Chirgwin JM, Yin JJ, Burns S, Rankin WA, Grubbs BG, Dallas M, Cui Y, Guise TA (2002). Transforming growth factor-beta stimulates parathyroid hormone-related protein and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways. *J Biol Chem* 277:24571-24578.
- Kedzierski RM, Yanagisawa M (2001). Endothelin system: the double-edged sword in health and disease. *Annu Rev Pharmacol Toxicol*. 41:851-876.
- McCauley LK, Koh-Paige AJ, Chen H, Chen C, Ontiveros C, Irwin R, McCabe LR (2001). Parathyroid hormone stimulates fra-2 expression in osteoblastic cells in vitro and in vivo. *Endocrinol* 142:1975-1981.
- Mundy GR (2002). Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat Rev Cancer*. 2:584-593.
- Mundy GR (2003). Endothelin-1 and osteoblastic metastasis. *Proc Natl Acad Sci U S A*. 100:10588-10589.
- Nelson JB, Carducci MA (2000). Small bioactive peptides and cell surface peptidases in androgen-independent prostate cancer. *Cancer Invest*. 18:87-96.
- Nelson JB, Nabulsi AA, Vogelzang NJ, Breul J, Zonnenberg BA, Daliani DD, Schulman CC, Carducci MA (2003). Suppression of prostate cancer induced bone remodeling by the endothelin receptor A antagonist atrasentan. *J Urol*. 169:1143-1149.
- Ruchon AF, Marcinkiewicz M, Ellefsen K, Basak A, Aubin J, Crine P, Boileau G (2000). Cellular localization of neprilysin in mouse bone tissue and putative role in hydrolysis of osteogenic peptides. *J Bone Miner Res*. 15:1266-1274.
- Sato K, Onuma E, Yocum RC, Ogata E (2003). Treatment of malignancy-associated hypercalcemia and cachexia with humanized anti-parathyroid hormone-related protein antibody. *Semin Oncol*. 30(5 Suppl 16):167-173.
- Schluter KD, Katzer C, Piper HM (2001). A N-terminal PTHrP peptide fragment void of a PTH/PTHrP-receptor binding domain activates cardiac ET(A) receptors. *Br J Pharmacol*. 132:427-432.
- Thalmann GN, Anezinis PE, Chang SM, Zhou HE, Kim EE, Hopwood VL, Pathak S, von Eschenbach AC, Chung LW (1994). Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. *Cancer Res* 54:2577-2581.

Wang X, Tokuda H, Hirade K, Kozawa O, 2002. Stress-activated protein kinase/c-Jun N-terminal kinase (JNK) plays a part in endothelin-1-induced vascular endothelial growth factor synthesis in osteoblasts. *J Cell Biochem* 87:417-423.

Wu HC, Hsieh JT, Gleave ME, Brown NM, Pathak S, Chung LW (1994). Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. *Int J Cancer* 57:406-412.

Yin JJ, Mohammad KS, Kakonen SM, Harris S, Wu-Wong JR, Wessale JL, Padley RJ, Garrett IR, Chirgwin JM, Guise TA (2003). A causal role for endothelin-1 in the pathogenesis of osteoblastic bone metastases. *Proc Natl Acad Sci U S A*. 100:10954-10959.

APPENDICES:

Revised (2/2003) SOW (3pp).

Three abstracts (1p each) from oral presentations at international meetings, fall 2003.

Proposal Title: **PSA converts parathyroid hormone related protein (PTHrP) from an osteolytic to an osteoblastic factor: role in bone metastasis**

STATEMENT OF WORK, revised February 2003:

The actual numbered tasks remain unchanged, as do the **Specific Aims**.

Additions to, changes in, or completions of individual tasks are indicated in **bold**. The time frame in Virginia is now:

03/01/2003-12/31/2003 = *months 1-9* (in italics)
01/01/2004-12/31/2004 = *months 10-21* (in italics)
01/01/2005-12/31/2005 = *months 22-33* (in italics)

Months not italicized are the months from the original SOW.

Please note that **Proposed Animal Use Is Unchanged**

For Specific Aim 1):

Task 1: Reclone prohK2 & proPSA cDNAs into pcDNA vectors, test DNAs by transient transfections into 293 cells and assay as in task 4, below (months 1-3). **COMPLETE**.

Task 2: Isolate stable single cell lines with DNAs from task 1 (months 4-12). **Complete for proPSA**. Cell lines for hK2 remain to be isolated (*months 1-9*).

Task 3: Isolate stable double (hK2+PSA) cell line with DNAs from task 1 and hK2 stable clone from task 2 (months 13-24; now *months 6-21*).

An additional section to task 3 has been added. The Isaacs' laboratory has just published (Denmeade et al, March 2003) detailed characterization of a series of prostate cancer cell lines for their expression of both hK2 and PSA. These lines are LAPC-4, CWR22Rv1, MDA PCA-2b, LNCaP, and C4-2B (derived from LNCaP), all of which are available to us through the resources of the NCI-supported UVa Cancer Center. We propose to assay these cell lines for PTHrP expression and secretion by three assays. First RNAs will be prepared from the 5 cell lines or xenografts and analyzed by standard RT-PCR for PTHrP gene expression. Second, conditioned media from the cells will be assayed for content of intact PTHrP with the Nichols Institute two-site IRMA. Third, the same media samples will be assayed with a single-site competition RIA, which employs a high-affinity antibody that recognizes the amino-terminus of PTHrP (residues 1-7), from Phoenix, Inc, which has been used successfully by our UVa colleague, Dr. S.J. Parsons. This assay will detect all cleaved forms of PTHrP. We expect that at least some of these cell lines, which make high amounts of active PSA and hK2, may cleave PTHrP efficiently. Media from these lines will be negative with the Nichols ELISA and positive with the Phoenix RIA.

Task 3a: **Screen 5 cell lines for PTHrP expression** (*months 1-9*). If none are positive, we will transfect a PTHrP expression DNA (already available and tested) into one of the hK2+, PSA+ lines. Resultant clones would then be screened with the three assays for PTHrP.

Task 3b: **Isolate PTHrP+ clones for a prostate cancer hK2+, PSA+ cell line** (*months 10-21*).

Task 4: Screen clones from tasks 2&3 for enzymatic activity and protein expression (months 6-12, 18-24, now *months 1-21*)

Task 5: Immuno-affinity purify PTHrP fragments from media of cell lines from tasks 2&3 and analyze

fragments by HPLC/mass spectrometry (months 13-18, 25-30, *now months 9-15, 25-30*). **Analysis of fragments from cell lines newly identified from additional subtasks 3a & 3b now included.**

For Specific Aim 2):

Task 6: Prepare synthetic peptides (months 1-3). **COMPLETED.**

Task 7 Test synthetic peptides on osteoclast formation in marrow cultures (months 4-9). **COMPLETED.**

Task 8: Test synthetic peptides on new bone formation in calvarial cultures (months 6-24). **Substantially completed.** Final experiments as originally proposed (*months 1-6*).

Additional section to task 8. Neonatal mouse calvarial organ cultures are slow and expensive. Now that we have demonstrated the essential correctness of the original hypothesis in organ culture, we will obtain more accurate dose response data using a well-characterized mouse pro-osteoblastic cell line, MC3T3-E1. Twenty-three published papers have tested the ET-1 receptor responsiveness of these cells, and others have shown PTHrP responsiveness (McCauley et al, 2001). Thus, the cells express receptors for both peptides and respond to them. The cells will differentiate into mature osteoblastic cells in culture when treated with ascorbate and form mineralized nodules, as well as express late markers, such as osteocalcin. We will carry out detailed dose responses of the peptides using these cells. We are presently determining the most convenient marker of differentiated response to assay (such as VEGF promoter-luciferase activity (Wang et al, 2002), alkaline phosphatase secretion, or osteocalcin production.)

Task 8a: Test peptides on MC3T3-E1 cells (*months 10-24*).

Task 9: Perform tasks 7, 8, and 8a on samples from task 5 (months 19-20, 31-32, *now months 9-15 & 37-30*).

For Specific Aim 3):

Task 10: Carry out pilot animal experiment for PC3/ev and PC3/hK2 cell lines from Aim 1) in metastasis model with 8 mice (months 15-20, *now months 10-15*).

Task 11: Carry out full animal experiment for the final four cell lines from Aim 1 in metastasis model (n=12 mice/group) and intramuscular tumor growth rate model (n=6 mice/group) with 72 mice total (months 25-30, *now months 25-30*). **Alternatively, this task could be carried out with one of the cell lines identified in task 3a or 3b.**

Task 12: Carry out detailed histomorphometric analysis of the bones and soft tissues of the 8 mice from task 10 (bones only, months 21-24, *now months 16-21*) and the 48 metastasis model mice (48+288 bone histology blocks) from the previous task (months 31-34, *now months 30-33*).

Task 13: Perform statistical analysis of data from animal experiments and prepare manuscripts for publication (months 11-12, 23-24; 35-36, *now months 3-6, 20-21 and 32-33*).

Summary of the original 01 year progress report,

submitted during the relocation to University of Virginia effective 10/01/02:

The aims, tasks, and the schedule remain essentially unchanged from those originally proposed:

Task 1: complete

Task 2: underway and expected to be completed by 6/30/03

Task 3: to be initiated after transfer to Virginia

Task 4: assay procedures already established. Experiments to be initiated after transfer to Virginia

Task 5: affinity columns of immobilized anti-PTHrP N-terminal 3F5 monoclonal antibody already made. They are presently being tested and will be used as proposed after transfer to Virginia

Task 6: completed for PTHrP peptides 1-16, 1-20, 1-23, and 1-34

Tasks 7 & 8: completed for PTHrP peptides 1-16 and 1-34, underway for 1-20 and 1-23. Task 7 now uses an assay that combines the cultures of task 8, but is otherwise unchanged

Tasks 9-13: will be carried out according to the originally proposed schedule following transfer to Virginia

At this time of the transfer from UTx to UVa, all of the proposed tasks were on schedule. We anticipate being able to keep to the original tasks and timetable despite the inevitable dislocations and delays associated with a cross-country move of laboratory and personnel.

References added for February 2003 revision:

Denmeade SR, Sokoll LJ, Dalrymple S, Rosen DM, Gady AM, Bruzek D, Ricklis RM, Isaacs JT. Dissociation between androgen responsiveness for malignant growth vs. expression of prostate specific differentiation markers PSA, hK2, and PSMA in human prostate cancer models. *Prostate* 54:249-257, 2003.

McCauley LK, Koh-Paige AJ, Chen H, Chen C, Ontiveros C, Irwin R, McCabe LR. Parathyroid hormone stimulates fra-2 expression in osteoblastic cells in vitro and in vivo. *Endocrinol* 142:1975-1981. 2001.

Wang X, Tokuda H, Hirade K, Kozawa O. Stress-activated protein kinase/c-Jun N-terminal kinase (JNK) plays a part in endothelin-1-induced vascular endothelial growth factor synthesis in osteoblasts. *J Cell Biochem* 87:417-423, 2002.

American Society of Bone & Mineral Research 25th Annual Meeting, Minneapolis, Minn.

Oral presentation # 1093, Cancer & Bone I, September 21, 2003

PTHrP Stimulates New Bone Formation by Molecular Mimicry of Endothelin-1

K.S. Mohammad, T.A. Guise, J.M. Chirgwin

University of Virginia, Charlottesville VA 22903

Abstract:

Prostate cancer metastases to the skeleton are frequently osteoblastic, despite abundant expression of the osteolytic factor parathyroid hormone-related protein, PTHrP. We propose a new explanation for this paradox: N-terminal fragments of PTHrP stimulate new bone formation by activating the endothelin A (ET_A) receptor. Endothelin-1 (ET-1) is a 21 aa peptide which potently stimulates new bone formation via the ET_A receptor. ET-1 residues 6-9 [LMDK] and PTHrP residues 8-11 [LHDK] share strong sequence homology. We tested PTHrP 1-16 in an ex vivo assay. Hemi-calvariae [n=4] of 4 day old mice were cultured on grids in medium + 0.1% BSA and test factor for 4 or 7 days. They were then fixed, decalcified, and embedded in paraffin. Sections were cut parallel to the sagittal suture, stained with H&E, and analyzed by computerized histomorphometry to determine new bone area and osteoblast number. Inclusion of 100nM PTHrP 1-16 caused a 3-fold stimulation of new bone area at 4 days (0.0095 vs 0.0035mm² for untreated control, p<0.05) and 7 days (0.014 vs 0.0035mm² for untreated control, p<0.001). Osteoblast numbers were correspondingly increased. The response was equivalent to that caused by 100nM ET-1 positive control. There was no additional response to 1000nM PTHrP 1-16 or to the combination of 100nM each PTHrP 1-16 and ET-1. The effects on new bone area and osteoblast number were blocked by the inclusion of 0.01mM ABT627, a selective ET_A receptor antagonist, which did not block new bone formation stimulated by FGF-2. We found similarly strong anabolic responses to 25nM PTHrP 1-20 and 1-23, while PTHrP 1-34 instead caused extensive osteolysis. Our data demonstrate that N-terminal peptides of PTHrP exert potent anabolic effects on bone via the ET_A receptor. The sequence of PTHrP 18-23, RRRFF, is cleaved by prostate-specific antigen. Proteolysis at this site frees residues 8-11 to bind to ET_A, providing a molecular explanation for the osteoblastic phenotype of PTHrP-positive prostate cancer bone metastases. The mimicry of ET-1 by PTHrP N-terminal peptides suggests that ET_A receptor antagonists could be effective in treating prostate cancer, both against the actions of endothelin itself and against the anabolic effects of PTHrP fragments.

The IVth International Conference on Cancer-Induced Bone Disease, San Antonio TX
Oral Presentation #16, 7 December 2003

PTHrP Fragments Stimulate New Bone Formation by Binding to the Endothelin A Receptor

K.S. Mohammad, T.A. Guise, and J.M. Chirgwin

University of Virginia, Charlottesville VA 22903

Abstract:

Prostate cancer metastases cause disorganized new bone formation, despite expressing the bone-destructive factor, PTHrP. We report a molecular basis for this paradox: proteolytic fragments of PTHrP stimulate bone cells via the endothelin A receptor (ET_AR). Residues 6-9 [LMDK] of ET-1 and PTHrP residues 8-11 [LHDK] share strong sequence similarity. PTHrP is cleaved at residues 22 and 23 by the serine protease, prostate-specific antigen (PSA). The fragments generated by PSA are too short to activate the PTH1 receptor but stimulate new bone by activating the ET_AR.

We tested PTHrP N-terminal peptides on bone formation in 4-day neonatal mouse calvariae. PTHrP(1-16) (100nM) caused a 2.5-fold stimulation of new bone area (0.014 vs 0.0035mm² for untreated control, $p < 0.001$). Osteoblast numbers were correspondingly increased. The response was equivalent to that caused by the established osteoblastic factor ET-1 (100nM). PTHrP(1-20) (25nM) also increased bone formation. The action of PTHrP(1-16) was blocked by ABT627 (0.01mM), a selective ET_AR antagonist, which did not inhibit new bone formation stimulated by FGF-2. PSA cleaves PTHrP after residue F23, allowing residues 8-11 to bind to ET_AR. We found a strong bone anabolic response to PTHrP(1-23) (1nM; $p < 0.01$ vs control), while PTHrP(1-34) instead caused extensive osteolysis.

Structural mimicry of ET-1 by PTHrP peptides provides a molecular basis for the osteoblastic phenotype of PTHrP-positive prostate cancer bone metastases and suggests that ET_AR antagonists should be effective against both the action of ET-1 and the anabolic effects of PTHrP fragments, in treating osteoblastic bone metastases due to prostate cancer.